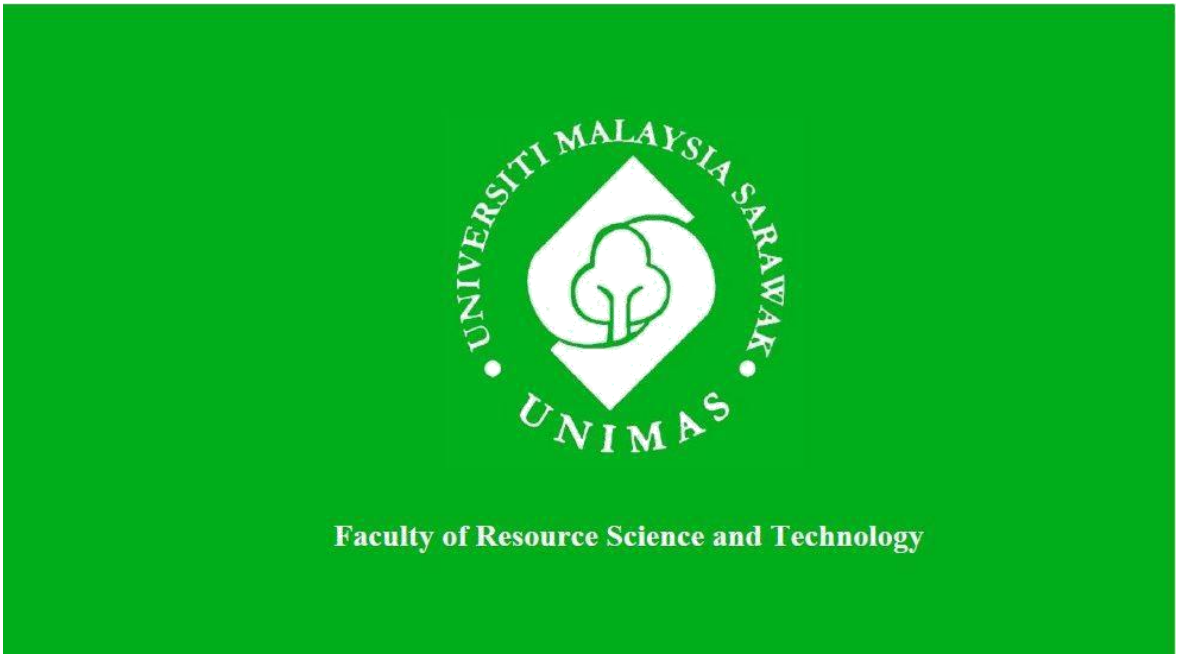

LIPID ACCUMULATION IN *Lipomyces starkeyi* MV-4 CULTURED IN GLUCOSE MEDIA

Ummi Syahida binti Zamri (39213)

Bachelor of Science with Honours
(Resource Biotechnology)
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DECLARATION

I hereby declare that this Final Year Project 2014/2015 dissertation, submitted to Universiti Malaysia Sarawak as a partial fulfillment of the requirement for the degree of Bachelor of Resource Biotechnology. I also certify that the work described here is entirely my own except for the quotations and citations whose resources are appropriate cited in the references.

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(Ummi Syahida binti Zamri)

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List of Abbreviations

L. starkeyi	*Lipomyces starkeyi*
FAME	Fatty Acid Methyl Ester
ATP	AdenosineTriPhosphate
LB	Lipid Bodies
TAGs	Triglycerides
OD	Optical Density
RT	Room Temperature
OY	Oily Yeast
NOY	Non-Oleaginous Yeast
SCO	Single Cell Oil
Abs	Absorbance
%	Percentage

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Lipid Accumulation in *Lipomyces starkeyi* Mutant Cultured Glucose Media

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ABSTRACT

Nowadays, biodiesel has gained a lot of attention and the demand for it also increased. The conventional method to produce biodiesel from plant oil has some limitations. It leads to the discovery of *Lipomyces starkeyi* MV-4 (*L. starkeyi* MV-4) that could accumulate lipid up to 70% of its dry biomass weight. The aim of this study was to increase the lipid produced when cultured in different concentration of glucose. Two different glucose concentrations that were used were 2.5% (w/v) and 5.0% (w/v). The optimum growth of *L. starkeyi* MV-4 has been identified on sixth day. The highest dry biomass weight and lipid produced when cultured in 2.5% (w/v) of glucose was at day eight with 17.23 g/L and 3.73 g/L respectively. When cultured in 5.0% (w/v) of glucose, the highest dry biomass and lipid accumulation was at day seven with 17.90g/L and 4.36g/L respectively. The phenol- sulphuric test was performed to determine the glucose profile. The residual of glucose for 2.5% (w/v) and 5.0% (w/v) of glucose were 0.06g/L and 0.29g/L respectively.

Keywords: *Lipomyces starkeyi* MV-4, glucose, phenol-sulphuric test, lipid accumulation

Pada zaman kini,biodiesel mendapat banyak perhatian dan permintaan terhadapnya juga meningkat. Cara konvensional untuk menghasilkan biodiesel dari minyak tumbuhan mempunnyai had terhadap alam sekitar. Ia telah membawa kepada penemuan Lipomyces starkeyi MV-4 (L. starkeyi MV-4) yang boleh mengumpul lemak sehingga 70% daripada biojismnya. Tujuan projek ini adalah untuk meningkatkan penghasilan lemak apabila di biakkan dalam kepekatan glukosa yang berbeza. Dua kepekatan glukosa yang digunakan adalah 2.5% (w/v) dan 5.0% (w/v). Pertumbuhan yang optimum bagi L. starkeyi MV-4 telah dikenalpasti iaitu pada hari keenam. Biojisim dan penghasilan lemak yang paling tinggi apabila dibiakkan di dalam 2.5% (w/v) glukosa adalah pada hari kelapan dengan masing-masing17.23g/L dan 3.73g/L. Apabila di biakkan di dalam 5.0% (w/v) glukosa, biojisim dan penghasilan lemak paling tinggi adalah pada hari ketujuh iaitu 17.90g/L dan 4.36 g/L. Ujian fenol-sulfurik telah dilakukan untuk mengetahui profil glukosa. Baki glukosa di dalam 2.5% (w/v) dan 5.0% (w/v) glukosa adalah 0.06g/L dan 0.29g/L.

Katakunci: *Lipomyces starkeyi* MV-4, glukosa, ujian fenol-sulfuric, penggumpulan lemak

CHAPTER 1

INTRODUCTION

Diesel is one of the components in fossil fuel. However, the over-use of diesel could produce greenhouse gases such as carbon dioxide gases which are the major elements leading to global warming. Hence, due to increase in demand and source limitation, biodiesel is introduced as a substitute for diesel fuel (Wild *et al*., 2010).

Biodiesel is a diesel fuel substitute that is extracted from renewable biomass. Biodiesel can be produced from plant oils, animal fats and microorganisms. Traditionally, biodiesel is produced from plant oils which were transesterify with methanol (Dai *et al.*, 2007). However, production of biodiesel from plant oils is not suitable due to the quality of tillable land (Li *et al.*, 2008) and competition with food production (Wahlen *et al*., 2012). Furthermore, the increase in animal fats prices due to the increase in animal feed makes it not suitable as biodiesel feedstock (Li *et al.*, 2008). Hence, oleaginous microorganisms have been introduced as good candidates for biodiesel feedstock.

Oleaginous microorganisms can accumulate lipid up to 20% of its cell dry weight. Oleaginous microorganisms have the ability to utilize different carbon source (Ageitos *et al.*, 2011). In this study, *Lipomyces starkeyi* was used. This type of yeast has the ability to produce lipid up to 70% of its cell dry weight (Wild *et al*., 2010). *L. starkeyi* can utilize different types of carbon as its sole carbon and it is flexible in terms of culture conditions (Ageitos *et al*.,2011). However, *L. starkeyi* is still not economically practical because of the limitations in the wild-type strains (Ageitos *et al.*, 2011). Therefore, in our research, we used *L. starkeyi* mutant in an attempt to produce more lipids in the fungal cells.

The *L. starkeyi* mutant was cultured in modified media consists of glucose, (NH_4) SO_4, yeast extract, $Na_2HPO_4.7H_20$, KH_2PO_4, $MgSO_4$. $7H_20$, $CaCl_2.2H_20$, $FeSO_4$, $ZnSO_4.H_20$ and $CuSO_4$ supplied with 2.5% (w/v) and 5.0% (w/v) of glucose and in separated Schott bottles. The temperature that was be used is room temperature (± 27°C). In this experiment, glucose served as carbon source for *L. starkeyi*. The total carbohydrate that would be consumed by *L. starkeyi*MV-4 was tested using phenol-sulphuric test.

The objectives in this research are:

1) To optimize the growth and lipid production of *L. starkeyi* MV-4.

2) To measure the amount of lipid produced by *L. starkeyi* MV-4 cultured in 2.5% (w/v) of glucose medium.

3) To measure the amount of lipid produced by *L. starkeyi* MV-4 cultured in 5.0% (w/v) of glucose medium

CHAPTER 2

LITERATURE REVIEW

2.1 Biodiesel

Biodiesel is the substitute of petroleum-based-diesel-fuel and biodiesel is the diesel fuel that is produced from vegetables and animal based feedstock (Wild *et al*., 2010). It consists of alkly ester of fatty acids or triglycerides. Triglyceride is the combination between glycerol and three fatty acids. Glycerol contains 3 hydroxyl group (OH) while each fatty acid has a group of carboxyl group (-COOH). The reaction between hydroxyl group and carboxyl group would form ester bond. The low barrier of C-O-C caused the rotation for the esters group flexible. The ester bond also has a low melting and boiling point (Mostafa *et al*., 2013).

Biodiesel is non toxic and biodegradable. It is also known as environmental friendly because it has low emission profiles (Ma and Hanna, 1999).There are two methods in producing biodiesel which are first-generation and second generation. First generation is the production of biodiesel from plant oils. Second generation is produced from the fermentation of waste, non-lignocellulosic and lignocellulosic materials, waste oils, animal oils and non-fertile soils (Sims *et al.,* 2010).

Conventionally, triglyceride is produced from soybeans oil with the addition of alcohol and acid or base catalyst. This process is known as transesterifications which could produce Fatty Acid Methyl Ester (FAME) (Wahlen *et al*., 2012). Basically, biodiesel can be derived from 3 sources which are plants oil, animal fat and microorganisms (Meng *et al.*, 2008).

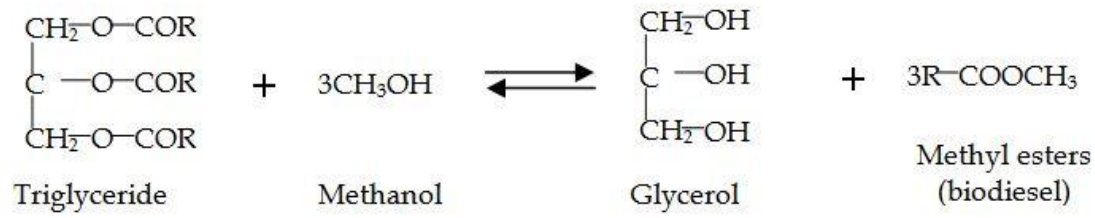

Figure 1.Transesterification process shows that the triglyceride is transesterify with methanol (alcohol) producing glycerol and biodiesel or methyl ester. Figure retrieved from www.impergam.sk/zaklad_Bio.

Plant oils that involve in the production of biodiesel are rapeseed, oil palm, soybeans, and many possible crops (Santamauro *et al*., 2014). However, the practical used of plant oils raises critical issues on the decreasing in quality of land that is needed to plant the crops could affect the quality of the crops produced (Li *et al*., 2008). In addition, it also competes with the food production (Wahlen *et al.*, 2012). Animal fat is also not a good biodiesel feedstock due to economical reasons (Meng *et al*., 2008). Hence, oleaginous microorganisms stand out as a potential feedstock provider.

2.2 Oleaginous microorganisms

Oleaginous microrganisms are the microorganisms that capable of producing oil. According to Ageitos *et al*. (2011) these microorganisms could produce lipid up to 20% of their weight. The benefit of using oleaginous microorganisms is, it could helps in aerobically produced lipid from organic matters residual (Xue *et al*., 2006).

Oleaginous yeasts (OY) are known producers of single cell oil (SCO). SCO produced from this organism are triacylglycerides (TAG) that have long-chain of fatty acids and have similar properties with plant oils. TAG acts as source of energy and it assist in phospholipid membrane formation (Meng *et al*., 2008). According to Ageitos *et al*. (2011), the differences between OY and non-oleaginous yeast (NOY) is NOY would stop grow

when the nitrogen is exhausted. The mithochodrial citrate level also different between OY and NOY in which it is higher up to three to four times in OY. Other than that, OY also has cytosolic citrate lyase in which it used ATP and magnesium, but it could used cobalt and manganese. OY also has AMP N-dependent dehydrogenase in which it helps in lipid accumulation (Ageitos *et al*., 2011).

The duplication rate of yeast is less than 1 hour and it is easy to culture compared to other microalgae. Other than that, certain oily yeast also has the ability to produce lipid up to 80% of their dry weight, while utilizing different carbon source including the lipid present in media (Ageitos *et al.*, 2011). OY also utilizes various its carbon sources from waste substrate thus the cost to culture this microorganism is low (El-Fadaly *et al*., 2009).

There are four groups of oleaginous microorganisms that capable of producing biodiesel which are bacteria, algae, filamentous fungi and yeast (Kitcha and Cheirsilp, 2011). The genera of oleaginous yeast are *Yarrowia, Candida, Rhodotorula, Rhodosporium, Crytococcus, Trichosporon* and *Lipomyces* (Ageitos *et al*., 2011). The specific name for the most preferable candidates for production of lipid are *Cryptococcus albidus, Rhodosporidium toruloides, Rhodotorula glutinis, Lipomyces starkeyi* and *Yarrowia lipolytica*. These microorganisms are capable of producing intracellular lipid more than 20% of its cell dry weight (Tapia *et al*., 2012).

In this study, *L. starkeyi* MV-4 was used. According to Tapia *et al*. (2012), the purpose of using the mutate strain compared to the wild-type strain is because the wild-type strain is not economically viable due to the wild-type productivity limitation. The wild-type strain also reported as refractory species to conventional genetic engineering (Tapia *et al*., 2012).

2.3 Factors affecting lipid accumulations in oleginous yeast

Lipid accumulations occur when yeast is cultured under high amount of carbon source but in limited source of nitrogen. This is due to the nutrient imbalance that helps in triggering the accumulation of lipid because the remaining substrate would be assimilated by the yeast's cells hence convert it into fat for storage (Ageitos *et al.*, 2011). The fat that accumulated could be extracted to produce biodiesel. In addition, the accumulations of lipid also affected by other factors such as the present of microelements and inorganic salts in media. These elements help in ATP (Adenosine Triphosphate) citrate lyse which important in lipid production (Ageitos *et al.*, 2011).

According to Ageitos *et al*. (2011), the nitrogen limiting condition would cause the AMP N-dependent dehydrogenase to produce low AMP (AdenosineMonoPhosphate) but increase in isocitrate in which it would be converted to citrate by enzyme mithochondrial aconitase. The citrate would the be transport by citrate transporter in cytoplasm to be converted into acetyl-CoA. Acetyl-CoA would be used in lipid synthesis (Ageitos *et al*., 2011).

2.4 *Lipomycesstarkeyi*

L. starkeyi is one of the members of *Saccharomycetales* and considered as true inhabitant of soil which have a worldwide distribution (Anschau *et al.*, 2014). *L. starkeyi* have the ability to accumulate lipid up to 70% of its dry weight (Wild *et al*., 2010). It also has a high flexibility in utilization of carbon source and culture environment. Other than that, fatty acid produced by *L. starkeyi* is almost similar to the vegetable oil (Tapia *et al.*, 2012). According to Wild *et al.* (2010), *L. starkeyi* need a high ratio of carbon to nitrogen in order

to optimize the production of lipid. The lipid bodies (LB) of *L. starkeyi* will receive the excess carbon source in the form of triglycerides (TAGs) (Ageitos *et al.*, 2011)

2.5 Phenol-sulphuric test

Phenol-sulphuric test is the quantitative assays which often used in estimation of carbohydrate. This test could detect the presence of neutral sugar in oligosaccharides, proteoglycan, glycolproteins and glycollipids (Albalasmeh *et al*., 2013).When phenol-sulphuric is added, the glucose that presence in samples would dehydrate thus forms hydroxymethyl furfurax. It would yield a yellow-brown product and the OD could be checked at 490 nm (Albalasmeh *et al*., 2013).

2.6 Lipid extraction

Lipid extraction is the removal of non-lipid contaminants. There various choice of lipid extraction which is the usage of chloroform: methanol, hexane propane and many more. However, chloroform: methanol increase many side effect to human health in which chloroform could cause tumour when tested in animal whereas, methanol impaired the visual system (Hara and Radin, 1987).

According to Hara and Radin (1987), there are several factors that must be considered in choosing correct solvents for the extraction of lipids which are volatility, toxic free, can form two phase system with water, can extract the non-lipid components, low price, can extract different classes of lipids, ultra-violet transparency, can be monitored in low wavelength region.

Based on the characteristic that required, the combination of hexane: isopropanol is the most suitable candidates. Although, hexane could cause neurotoxin when in high

concentration but through the metabolism to 2,5-diketo compound, it is safe to be used in laboratory. When performed lipid extraction, the lipid contains less non-lipid components and it can dry up to perform in chromatography column (Hara and Radin, 1987). The combination of hexane:isopropanol also has a lower toxicity compared to 'Bligh and Dyer' (B/D) method (Guckert and White, 1988).

Isopropanol act to inhibit phospholipase D and it helps to retain the lipid in its native state. Other than that, hexane: isopropanol combination only extracts a small amount of non-lipids and less pigment compared to the combination of chloroform: methanol. Hence, it is better to use hexane: isopropanol in the extraction of lipid (Guckert and White, 1988).

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

1. Yeast Malt Broth (YM Broth, Sigma-Aldrich, Inc., U.S.A)
2. *Lipomyces starkeyi* MV-4
3. 2.5% (w/v) and 5.0% (w/v) of glucose (EeSyn, Malaysia*)*
4. 80% (w/v) of Glycerol stock (HmbG, Germany)
5. 5% Phenol (Nacalai Tesque, Japan)
6. Hexane (Reagents, USA)
7. Isopropanol (Amresco, USA)
8. Ammonium sulfate (Systerm, ChemAR®, Poland)
9. Potassium dihydrogen phosphate (Systerm, ChemAR®, Poland)
10. Magnesium sulfate (Bendosen Laboratory Chemica, Norway)
11. Calcium-2-hydrate (Hamburg chemical Ltd.,Germany)
12. Yeast extract (Conda Pronadisa,Spain)
13. Glass bottle 2 L (SCHOTT, Duran®, Germany)
14. Centrifuge (CR21G, Hitachi, Japan)
15. Vortex (VX-200, Labnet International Inc., U.S.A)
16. Incubator shaker(Ecotron, Infors HT, Switzerland)
17. Analytical Balancer (Adventurer™ pro Balancer, Ohaus Corporation, U. S. A)
18. Homogenizer (Superfine Homogenizer, Fluko®, China)
19. Light microscope (BX51, Olympus, Japan)

3.2 Glycerol stock

Yeast Malt Broth (YMB) (2.1 g) was added in 100 ml of distilled water before sending for autoclave. The *L. starkeyi* MV-4 strain mutant was pipetted into the YMB. The culture was incubated for 3 to 5 days to allow the growth. The growth of cell could be detected when the media change its color from clear yellow to cloudy. Furthermore, simple staining was performed to check whether the samples were contaminated or not. Then, the sample was streaked onto Rose Bengal Chloramphenicol (RBC) agar in order to obtain pure colonies of mutant for glycerol stock. A single colony of mutant from RBC agar was further inoculated into YMB. After 4 days, 800 µl of *L. starkeyi* MV-4 was pipette into glycerol stock and stored in freezer at -20 °C.

3.3 Propagation of cell

L. starkeyi MV-4 (17mL) was cultured into 1.8 L of modified media (Wild *et al*., 2010) in 2 L Schott bottle with 3 holes on the cap. For day 1 until day 8, empty falcon tubes were weighted earlier. After that, 150 mL of the cultured from Schott bottle was sucked out into falcon tubes. The sample was sent for centrifuge for 10 min, 5000 rpm at 4 °C. The supernatant was discarded. The sample was dried in the oven for 2 days. After that, the sample was weighted for its dry weight. Graph dry biomass weight (g/L) against sampling hour was plotted to determine the highest day to add the glucose for lipid accumulation step.

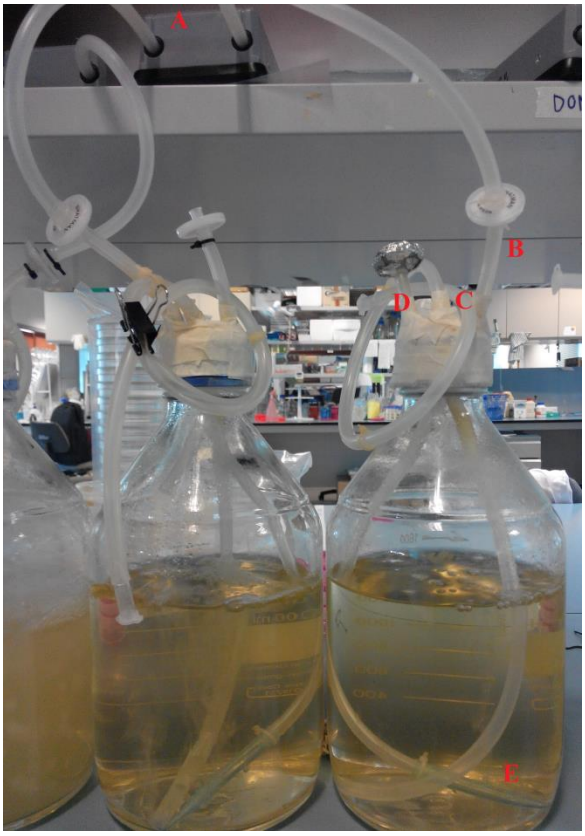

Figure 2.Propagation process for 1.8 L of modified media and glucose. A, sterile air from air pump B, Inlet (air in) C, Sampling D, Outlet (air out) E, pipette tip is used to dispersed the oxygen evenly.

3.4 Lipid accumulation stage for *L. starkeyi*MV-4

The *L. starkeyi* MV-4 (8.5 mL) culture was incubated for 6 days (optimum growth) at room temperature in 900 mL modified media. After 6 days, 900 mL of 10.0% (w/v) of glucose was added into the modified mediato achieve final concentration of 5% (w/v) in the 2 L Schott bottle. The culture was further incubated for 8 days. From day 1 to day 8, 150 mL of cultured was harvested in the Falcon tube. This step was repeated for 2.5% (w/v) of glucose.

3.5 Sampling biomass

The falcon tubes then were sent for centrifuge at 5000 rpm for 10 minute. The supernatant was discarded and the pellet was dried in the oven for 2 days at temperature 80˚C. After 2 days, the pellet was weightedfor its biomass dry weight.

3.6 Lipid extraction

The pellet was grind until fine particles was obtained and 15 mL of hexane: propanol in the ratio of 3:2 was added (Hara and Radin, 1987). The mixture was homogenized for 2 minutes. Then, the sample was sent for centrifuge at 5000 rpm for 10 minute. Beaker was weight earlier before transfer the supernatant. The supernatant was heated until the hexane and propanol solution have evaporated completely. The beaker containing oil was weighted again.

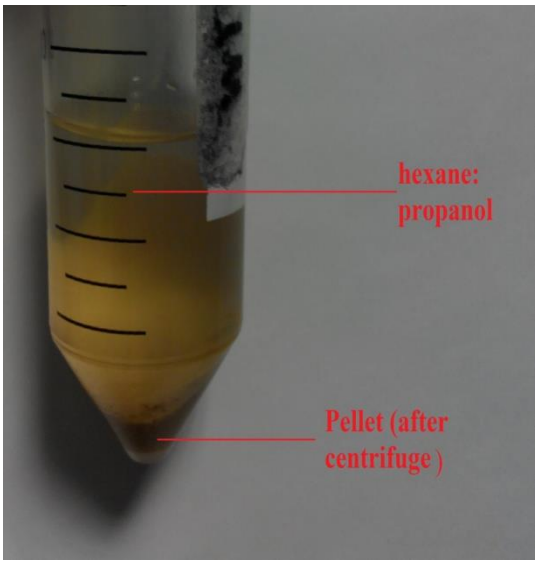

Figure 3. Lipid extraction method using the addition of hexane: propanol (3:2).

3.7 Phenol-sulphuric carbohydrate test

Phenol test was used to detect the amount of carbohydrate that is not consumed by *L. starkeyi* MV-4. First, the standard glucose for 5% (w/v) of glucose was prepared. Glucose (1 mL) was mixed with 4 mL of distilled water. The concentration for standard glucose is as below:

Table 1.Sugar percentage for glucose standard (5.0% (w/v).

Sugar (%)	Stock (mL)	Distilled water (mL)
5.0	2.0	0.0
4.0	1.6	0.4
3.0	1.2	0.8
2.0	0.8	1.2
1.0	0.4	1.6
0.5	0.2	1.8

For each test tube (triplicate), 0.2 mL sample was added before the addition of 0.2 mL of 5% phenol. Then, 1 mL of sulphuric acid and 5.6 mL of distilled water was added into each test tube. For each step, the test tubes need to be vortex to ensure the solution mix well. Then, 1 mL from each test tube was pipette into cuvette. The samples then view in spectrophotometer at 490 nm.

This step was repeated for *L. starkeyi* MV-4 samples. For *L. starkeyi* MV-4, 0.5 mL of sample was mixed with 2 mL of distilled water.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Propagation of *Lipomyces starkeyi* MV-4

Biodiesel production from oleaginous microorganisms' has received spot light due to its ability to accumulate lipid up to 20% of its cell dry weight. While, oleaginous yeast such as *Lipomyces starkeyi* is able to accumulate lipid up to 70% of its cell dry weight (Ageitos *et al.*, 2011).

In this study, mutated *L. starkeyi* MV-4 was used and it is hoped that the mutated strain could accumulate much higher lipid compared to the wild type strain. *L. starkeyi* MV-4 is mutated using ethidium bromide (EtBr) and the point of mutation is not known. A further study needs to be carried out to specify the point of mutation. The aims for the mutated *L. starkeyi* are to increase the lipid storage capacity and also to enhance the generation of rare fatty acid profiles (Zhao *et al*., 2008). EtBr would cause damaged at the microbial body in which it is the place for lipid storage. The damaged to the lipid bodies help to accumulate lipid.

The media used are modified media by Wild *et al*. (2010). The compositions of the media are glucose, yeast extract, ammonium sulfate, sodium hydrogen phosphate, potassium dihydrogen phosphate, magnesium sulfate and calcium-2-hydrate. Glucose acts as carbon source. Yeast extract serve as nutrient. Ammonium sulfate is inorganic salt which contain nitrogen and sulphur. *L. starkeyi* MV-4 consume the nitrogen from ammonium sulfate.

L. starkeyi could utilize several types of carbon sources namely glucose, xylose, ethanol and L-arabinose (Angerbauer *et al*., 2008). *L. starkeyi* MV-4 was grown in modified media

with glucose as its carbon source.*L. starkeyi* MV-4 was grown in limited ratio carbon to nitrogen. The limited nitrogen would enhance the accumulation of lipid in the lipid bodies. According to Wild *et al*. (2010), the amount of lipid content increase when the C:N ratio was changed to 61.2 g/L. Hence, the amount of lipid produced depends on the concentration of carbon and nitrogen. According to Ageitos *et al*. (2011), when the nitrogen is limited, the lipid synthesis would be increased and it would enhance the increase in glucose catabolism pathway through the pentose phosphate pathway. The lipid productiveness of the lipid produced depends on the amount of citric acid and the ability of its biochemical pathway to metabolize it (Ageitos *et al*., 2011). After the nitrogen limiting achieved, the amount of excess carbon from the high ratio of C:N might have been directed to the lipid accumulation stage (Azad *et al*., 2014). According to Azad *et al*. (2014), carbon and nitrogen initial concentration is important as it will affect the biomass constitute thus the amount of lipid accumulated.

Figure 4 shows the growth pattern of *L. starkeyi* MV-4. *L. starkeyi* MV-4 increased in their dry biomass weight.The propagation process was carried out for 192 hours to indicate the growth pattern from log phase until death phase. However, in this experiment, the death phase cannot be seen because at 192 h the cells of *L. starkeyi* MV-4 is still in stationary phase. A longer propagation time is needed to observe the death phase. At 0 to 24 h, the dry biomass weight is little in which it is still in the log phase. It is because the cells do not reproduce immediately in a new medium and also because the cells are in the phase of adapting to the new environment. For 48 until 192 h, the cells enter the exponential phase hence dry biomass obtained is increase in weight. It is due to the cells has adapt to the medium thus it could actively reproduce the new cells. The cells also actively consume the carbon source in the medium.